PAPER AND CRF COPIES OF SEQUENCE LISTING

In response to the Notice to Comply with Requirements for Patent Applications containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, please delete the existing paper copy of the Sequence Listing and replace it with the attached paper copy of a Substitute Sequence Listing. The amendment of SEQ ID NO:30 included in the Substitute Sequence Listing is supported as discussed above. I hereby state that the Substitute Sequence Listing does not include new matter. Attached is a copy of the Substitute Sequence Listing in computer readable form (CRF). I hereby state that the CRF copy of the Substitute Sequence Listing is the same as the attached paper copy of the Substitute Sequence Listing.

REMARKS

Applicant wishes to thank Examiner Terry McKelvey for the courtesy extended to their representative Nancy Vensko on July 10, 2000. The Interview Summary (Form PTOL-413) summarizes the discussions held at the personal interview. The present response to the outstanding Office Action includes the substance of the Examiner Interview.

Sequence Listing

As discussed above, Applicant has submitted a "courtesy copy" of the specification. The specification has been amended to correct a sequence listing error. In response to the Notice to Comply, a paper copy of a Substitute Sequence Listing is submitted, as well as a copy in CRF form.

Information Disclosure Statement

With regard to the 0 544 292 A2 reference only, the Examiner states that the information disclosure statement filed 11/19/99 fails to comply with 37 CFR 1.98(a)(3). It does not include a concise explanation of the relevance, as it is presently understood by the individual designated in 37 CFR 1.56(c) most knowledgeable about the content of the information, of each patent listed that is not in the English language. The result is

that the aforementioned not-in-the-English-language reference has been placed in the application file, but the information referred to therein has not been considered.

Supplemental Information Disclosure Statement

Enclosed is a Supplemental IDS and form PTO-1449 listing references that are also enclosed. Competitors brought to our attention Kaneda et al., Science 243, 375-378 (1989), Haensler and Szoka, Bioconjugate Chem 4, 85-93 (1993), Citovsky et al., Science 256, 1802-1805 (1992), and Rosenkranz et al., Experimental Cell Res 199, 323-329 (1992). The Kaneda et al. and Citovsky et al. journal articles were already cited in the previous IDS. However, Applicant has enclosed all 4 journal articles with the supplemental IDS for completeness. Two U.S. patents that recently issued are presented for consideration.

Claim Objections

The Examiner objected to Claims 12 and 13, and 15-20 because of various informalities. The claims have been amended to correct the informalities. Thus, Claims 12 and 13 are grammatically reformed and Claims 15-20 omit the redundant term.

Reissue Application

Applicant acknowledges the requirement under 37 CFR 1.178 that the original patent, or an affidavit or declaration as to loss or inaccessibility of the original patent, must be received before this reissue application can be allowed. The "ribboned" original patent is enclosed as required. The provision under 37 CFR 1.178 is thus satisfied.

No New Matter

The Examiner rejected Claims 17-20 under 35 USC 251 as being based upon new matter. Specifically, Claim 17 is drawn to a transfection vector in a kit, while Claim 18 is drawn to a transfection vector outside a cell (and Claims 19-20 at least partly depend on these independent claims). The Examiner took the position that the antecedent basis for these claims indicated by Applicant to be at column 1, lines 5-10 and Example 1 provides a description of the transfection vector but only in a kit or

present outside of a cell within the context of the specific example and not as a broader invention of a transfection vector in a generic kit or outside of a generic cell. Any objection or rejection of the specification or claims under 35 USC 112 or 251 is respectfully traversed.

The question presented is whether the patent specification would have allowed one skilled in the art to have understood the specification as describing the claimed invention, with all its claims limitations. Gentry Gallery Inc. v. Berkline Corp., 45 USPQ2d 1498, 1503 (Fed. Cir. 1998). A fair reading of column 1, lines 5-10 and Example 1 indicates antecedent basis for the claims limitations of a transfection vector existing in a kit or outside a cell. For instance, the heading under Example 1 says, "Forming the DNA-Polypeptide Complex and Transfecting Cells Therewith." Logically, if one transfects cells with a transfection vector, then the transfection vector is present initially outside the cells and subsequently inside the cells. It is also logical that if the transfection vector is present initially outside the cells, then the transfection vector exists in a container (until applied to the cells). Claims 17 and 18 simply claim the alternative where the transfection vector is present initially outside a cell (Claim 18) and contained in a kit (Claim 17).

This case is unlike Gentry Gallery, in which the court determined that the patent specification did not support a broad interpretation of where the control means should be located based on the unambiguous location of the control means on the fixed console. Here, the patent specification provides ample support for the breadth of an interpretation of where the transfection vector should be located. It does not unambiguously limit the location of the transfection vector. Instead, the patent specification generically describes forming a DNA-polypeptide complex and transfecting cells therewith thus making clear that the exact location of the transfection vector is to be expansively interpreted to include in a kit or outside a cell. Consequently, the Examiner is respectfully requested to withdraw the new matter rejection.

No "Double Patenting"

The Examiner objected to Claims 17 and 18 as being a substantial duplicate of Claim 15 under MPEP 706.03(k). Claim 17 is drawn to a transfection vector in a kit,

and Claim 18 is drawn to a transfection vector outside a cell. In contrast, Claim 15 is drawn to the transfection vector without the additional limitations regarding its exact location. The Examiner concludes that the exact location of the transfection vector does not impart any material distinction upon the transfection vector itself, that the claimed transfection vector of Claim 15 is identical to the transfection vector of Claims 17 and 18, and that therefore Claims 17 and 18 are substantial duplicates of Claim 15 in contravention of MPEP 706.03(k).

However, the Examiner has allowed "kit" claims before. Attached are USP 5,968,773, 5,948,883, and 5,869,258. The Examiner of this application is the same as the Examiner of those patents. Focusing on the '883 patent, a human CRM1 protein is claimed. Claim 1 claims the polypeptide. Claim 3 claims a kit comprising the polypeptide. The reason the claims in the '883 patent are not substantial duplicates of one another is because the patent is presumed valid, and thus the additional limitation regarding the exact location of the polypeptide in a kit must impart a material distinction upon the polypeptide itself.

Likewise, here, the additional limitation regarding the exact location of the transfection vector in a kit or outside a cell does indeed impart a material distinction upon the transfection vector itself, because the environment of use specifies a further limitation. Thus, the claimed inventions of Claims 17 and 18 are not identical to the claimed invention of Claim 15. Consequently, Claims 17 and 18 are not substantial duplicates of Claim 15, and MPEP 706.03(k) is observed.

Novelty and Nonobviousness

The Examiner rejected Claims 15-20 under 35 USC 102(e) as being anticipated by or 35 USC 103 as being unpatentable over USP 5,955,365 to Szoka, Jr. et al. The present invention as defined in new Claims 15-20 (using "closed" language) and new Claim 21 (adding a counterpart to pending Claim 8) is neither anticipated by nor unpatentable over Szoka, Jr. et al. Szoka, Jr. et al. describe a self-assembling polynucleotide delivery system comprising components aiding in the delivery of the polynucleotide to the desired address which are associated via noncovalent interactions with the polynucleotide (abstract). The components of this system include DNA-

associating moieties that interact in a non-covalent fashion with the polynucleotides and are covalently linked to the rest of a functional component, such as a subcellular-localization component, like a NLS (col. 13, lines 1-57). The DNA-associating moiety can be a polycation, including polylysine, polyarginine and poly (lysine-arginine) (col. 13, lines 53-57). Figure 1 shows one embodiment:

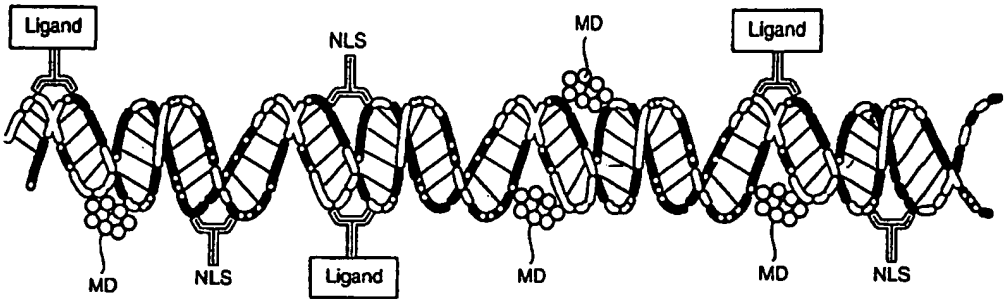

Figure 1 makes clear that the self-assembling polynucleotide delivery system of Szoka, Jr. et al. does not contemplate the transfection vector of Claims 15-21, where the transfection vector is defined as a synthetic polypeptide linked electrostatically to a DNA structural sequence, forming a DNA-polypeptide complex, the polypeptide consisting of a NLS. In Claims 15-21, the DNA-polypeptide complex is formed by a NLS linked to a DNA structural sequence by an interaction that is electrostatic (i.e., non-covalent). In contrast, as pictured above, the Szoka, Jr. et al. self-assembling polynucleotide delivery system is formed by a NLS linked to a DNA-associating moiety (fork in Fig. 1) by an interaction that is covalent. The DNA-associating moiety then interacts in a noncovalent fashion with the polynucleotide. Because there is no disclosure or suggestion to form a self-assembling polynucleotide delivery system through linking a NLS to polynucleotide by an interaction that is electrostatic (i.e., non-covalent), Szoka, Jr. et al. neither anticipates nor makes unpatentable Claims 15-21. There is no motivation to leave out the intermediary DNA-associating moiety (fork in Fig. 1), because Szoka, Jr. et al. does not teach the concept that a self-assembling polynucleotide delivery system can be achieved via non-covalent interactions of a

polynucleotide and a NLS. The achievement of Claims 15-21 is a surprising advance over Szoka, Jr. et al., because it accomplishes a self-assembling polynucleotide delivery system comprising components aiding in the delivery of the polynucleotide to the desired address which are associated via noncovalent interactions with the polynucleotide, where the component is better than a DNA-associating moiety covalently linked to a NLS; the component is the NLS itself.

Any disclosure or suggestion in Szoka, Jr. et al. to mediate transfection through use of a polylysine, polyarginine or poly(lysine-arginine)-DNA complex (Table 2) is distinguishable. Claims 15-21 require a transfection vector, where the transfection vector is defined as a synthetic polypeptide linked electrostatically to a DNA structural sequence, forming a DNA-polypeptide complex, the polypeptide consisting of a NLS. Table 1 in the present specification evidences that a NLS is not defined as a polylysine, polyarginine, or poly(lysine-arginine) sequence. There is no exception, not even the mouse c-abl nuclear localization signal, which has been corrected by amendment to indicate that this NLS is not defined as a polylysine, polyarginine, or poly(lysine-arginine) sequence, either. The Examiner did not raise such a prior art rejection, and there is no reasonable basis for it. In conclusion, Claims 15-21 distinguish over Szoka et al. by virtue of the closed claim terminology.

Allowable Subject Matter

Applicant acknowledges the allowability of Claims 1-14, now that the informalities are corrected.

New Claims 15-21 Have Antecedent Basis

In response to the Examiner's verbal request, Applicant indicates where in the patent specification antecedent basis lies for Claims 15-21. The patent specification must describe the precise embodiment being claimed, either "literally" or "figuratively." In re Alton, 37 USPQ2d 1578, 1583 (Fed. Cir. 1996). In that case, the Patent Office had established a case of lack of written description because example 5 in the patent specification had described one analog containing two modifications together. However, the patentee wished to claim an analog containing one of the modifications

separately. The Federal Circuit told the Patent Office that it needed to consider declaratory evidence that one of ordinary skill in the art would have interpreted example 5 as describing another two analogs containing each modification individually.

As indicated by inventor Gopal in the attached *Supplemental Declaration for Reissue Patent Application* ("Gopal Declaration"), one of ordinary skill in the transfection art at the time of the May 11, 1994 filing date of the present application would not have known that a nuclear localization signal (NLS) peptide could be useful as a transfection vector to mediate DNA transfection, whether the NLS peptide occurred independently or "bipartite," namely linked through a hinge to a polymeric chain of basic amino acids. Additionally, as indicated in the Gopal Declaration, one of ordinary skill in the tranfection art would have understood the discussion in the patent specification as an illustration of the use of a NLS peptide as a transfection vector to mediate DNA transfection, where the NLS peptide appears not only independently but also in "bipartite" form, that is linked through a hinge to polymeric chain of basic amino acids. Moreover, as indicated in the Gopal Declaration, the inventor himself regarded his invention to be the use of a NLS peptide as a transfection vector to mediate DNA transfection, such that when reduced to its most basic embodiment, the NLS peptide occurred independently and a mere preferred embodiment constituted the "bipartite" version where it was linked through a hinge to a polymeric chain of basic amino acids.

As detailed in the Gopal Declaration, the original claims were directed to the preferred embodiment, but this conclusion does not negate the support for each alternative individually located, for example, in the very first paragraph of the patent specification, which says, "More specifically, the present invention is directed to the use of a synthetic polypeptide, containing a nuclear localization signal, to complex with a DNA molecule and to facilitate its transportation and integration into the nuclear genome of a mammalian or other eukaryotic cell ..." Moreover, the patent specification reiterates at col. 7, last line of the first paragraph, which says, "There has been no suggestion heretofore, however, to use a NLS peptide to target a polynucleotide to the nucleus of a eukaryotic cell." Thus, the patent specification describes the precise embodiment now being claimed.

Additionally, the declaratory evidence shows that an extension of the invention is a ligand attached to the transfection vector (either with or without a hinge). Thus, original Claim 8 was patented, and new Claim 21 is presented by this reissue application. The patent specification at the paragraph bridging col. 8 and 9 supports this nuance of the invention.

As verbally agreed by the Examiner, the patent specification describes a transfection vector as "containing" a NLS peptide. The declaratory evidence shows that this word choice is appropriate, because it is meant to be consistent with both "open" and "closed" claim terminology. Using the open construction, the transfection vector is an alternative that may be added to by an unlimited number of unrecited elements, as, for instance, in the embodiment that contemplates the use of a NLS peptide as a transfection vector where the NLS peptide occurs in "bipartite" form, i.e., linked through a hinge to a polymeric chain of basic amino acids. Using the closed construction, the transfection vector is an alternative that may not be added to by other elements, as, for example, in the embodiment that contemplates the use of a NLS peptide as a transfection vector where the NLS peptide occurs independently. The word choice is fitting because it develops each alternative individually.

The transition words "comprising" and "consisting of" have standardized meanings, as opposed to the transition word "containing" "Comprising" is recognized as being "open" and "consisting of" is supposed to be "closed." Ex parte Davis, 80 USPQ 448 (1950). By discarding these standardized terms, Applicant was able to avoid having to choose between the accepted constructions, thus achieving a description of each alternative individually.

The dictionary meaning (attached) defines "contains" as "to consist of wholly or in part" or "comprise." Webster thus embraces both "open" and "closed" constructions. This result accords with the goal of developing each alternative individually.

While MPEP 2111.03 says the word "containing" should be given an open interpretation, MPEP 2111.01 appreciates that non-standardized claim terminology must be interpreted in light of the patent specification. Here, the declaratory evidence establishes that one of ordinary skill in the transfection art would have interpreted the word "containing" as conveying that the inventor had possession of each alternative

individually. Thus, the patent specification supplies antecedent basis for the precise embodiment now being claimed by artful use of the transition word "containing."

CONCLUSION

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration and withdrawal of all outstanding rejections are respectfully requested. Allowance of the claims at an early date is solicited. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 9/5/00

By: _____

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Application No.: 09/404,979

NOTICE TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with the requirements for such a disclosure as set forth in 37 C.F.R. 1.821 - 1.825 for the following reason(s):

☒ 1. This application clearly fails to comply with the requirements of 37 C.F.R. 1.821-1.825. Applicant's attention is directed to these regulations, published at 1114 OG 29, May 15, 1990 and at 55 FR 18230, May 1, 1990.

☐ 2. This application does not contain, as a separate part of the disclosure on paper copy, a "Sequence Listing" as required by 37 C.F.R. 1.821(c).

☒ 3. A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 C.F.R. 1.821(e).

☐ 4. A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 C.F.R. 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing."

☐ 5. The computer readable form that has been filed with this application has been found to be damaged and/or unreadable as indicated on the attached CRF Diskette Problem Report. A Substitute computer readable form must be submitted as required by 37 C.F.R. 1.825(d).

☐ 6. The paper copy of the "Sequence Listing" is not the same as the computer readable from of the "Sequence Listing" as required by 37 C.F.R. 1.821(e).

☒ 7. Other: The application lacks a statement that the CRF and paper sequence listings are the same and include no new matter.

Applicant Must Provide:

☒ An initial or substitute computer readable form (CRF) copy of the "Sequence Listing".

☒ An initial or substitute paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification.

☒ A statement that the content of the paper and computer readable copies are the same and, where applicable, include no new matter, as required by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.825(b) or 1.825(d).

For questions regarding compliance to these requirements, please contact:

For Rules Interpretation, call (703) 308-4216
For CRF Submission Help, call (703) 308-4212
For PatentIn software help, call (703) 308-6856

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PEPTIDE-MEDIATED GENE TRANSFER

BACKGROUND OF THE INVENTION

The present invention is directed to a methodology for highly efficient, stable integration of DNA into a eukaryotic genome. More specifically, the present invention is directed to the use of a synthetic polypeptide, containing a nuclear localization signal, to complex with a DNA molecule and to facilitate its transportation and integration into the nuclear genome of a mammalian or other eukaryotic cell, for example, in the context of producing cell lines with an extended life.

DNA-$CaPO_4$ co-precipitation was the first method developed to introduce genes into mammalian cells. ("Gene" in this regard denotes a structural DNA segment, i.e., a DNA that codes for a polypeptide, and comprehends oncogenes as well as DNAs coding for a known expression product.) The co-precipitation method was applicable only to certain cell types, however, and could not be used to introduce genes into a wide variety of cell lines, especially those of hematopoietic origin. Moreover, the stable gene transfer efficiency was rather low, on the order of 10^{-4} to 10^{-6}. McNally, M. A., et. al., *BioTechniques* 6: 8826 (1988); Yen, T. S. B., et. al., loc. cit. 6: 413 (1988).

Limits on introducing and expressing genes in cultured mammalian cells motivated a search for other, more efficient approaches to gene transfer. Methods were developed, for example, that utilized chemical agents which were positively charged and, hence, able to complex with negatively charged DNA molecules. Examples of such agents include DEAE dextran and various cationic lipid molecules. Cells treated with DNA complexes comprised of such an agent can lead to the introduction of the DNA into different mammalian cell lines. Mannino, R. J. et. al., *BioTechniques* 6: 682 (1988); Felgner, P. et al., *Proc. Nat'l Acad. Sci. USA* 84: 7413 (1987); Fraley, R. et. al., *Trend Biochem. Sci.* 6: 77 (1981); Holter, W. et. al., *Exp. Cell Res.* 184: 546 (1989); McCutchan, J. H. et al., *J. Nat'l Cancer Inst.* 41: 351 (1986); Chaney, W. C. et al., *Somatic Cell & Mol. Genet.* 12: 237 (1986).

The production of a gene product for only a short time period after transfection, usually from 48 to 72 hours, is called "transient expression." Many of the DNA-complexing agents reported heretofore, while useful in transferring a gene into mammalian cells, resulted in only transient expression of the introduced gene in a small fraction of the transfected cells. See, for example, Miller et. al., *Proc. Nat'l Acad. Sci., USA*, 76: 949 (1979); Oi et al., loc. cit. 80: 825 (1983).

In addition to giving poor results with respect to stable gene expression, transfer methods based on such DNA-complexing agents often were effective only with established cell lines, and did not work very well with primary cells isolated from various mammalian species. Other techniques therefore were needed to enhance gene transfer efficiency, to increase the variety of cell types capable of being transfected, and to effect stable gene transfer. Stable gene transfer is the ability of cells to maintain and express transfected DNAs in a stable manner, through integration of the transfected DNA into cell chromosomes.

Retrovital vectors, which were under development at about the same time seemed to be quite effective in transferring genes into different cell types. The use of such vectors was prompted by the elucidation of gene regulation in various murine and avian retroviruses. Two other developments led to the development of retrovirus-based gene

transfer vehicles. The first development was the identification of minimal sequences required for efficient packaging of viral particles in a cell line which produced the coat proteins and other structural components of the viral particle in trans. The cell lines that provided the structural components for virus development are called "packaging" cell lines. The second significant step in the establishment of retroviral vectors was the development of both ecotropic and amphotropic packaging cell lines, which aided the design of recombinant retroviral particles which could infect both murine and human cell lines.

Additional modifications of retroviruses were deemed necessary to address concerns that retroviral vectors could recombine in vivo to generate wild-type virus. Developments in this regard yielded a number of safe retroviral vectors which have been used to transfer genes into a variety of established mammalian cell lines, as well as into certain primary cells in a few instances. E. Gilboa et al., *BioTechniques* 4: 504 (1986); A. D. Miller et al., *Mol. Cell. Biol.* 6: 2895 (1986); H. Stuhlmann et al., loc. cit. 9: 100 (1989); A. D. Miller et al., *BioTechniques* 7: 980 (1989); J. A. Zwiebel et al., *Science* 243: 220 (1989).

Even though these vectors were effective with respect to various mammalian cells, there were many restrictions on a wider application of the retroviral gene-transfer technique. These limitations included (1) the size of exogenous DNA that can be inserted into a retroviral vector and (2) the use of only dividing cells for retroviral gene transfer. E. Gilboa, *BioTechniques*, supra (1986); A. D. Miller, supra (1986); H. Stuhlmann, et al., *Mol. Cell. Biol.* supra, (1986); A. D. Miller et al., *BioTechniques, supra* (1986); J. A. Zwiebel et al., supra (1989).

Other viruses have been used to generate recombinant viral vectors for gene transfer studies. Adenovirus, adeno-associated virus, herpes simplex virus, and even HIV have been employed as vectors to introduce genes into both established cell lines and primary cells. Some of these viral vectors are capable of transferring genes into non-dividing cells. R. J. Samulski, et al., EMBO J. 10: 3941 (1981); J. D. Tratschin, et al., *Mol. Cell. Biol.* 5: 3251 (1985); P. L. Hermonat, et al., *Proc. Nat'l Acad. Sci. (USA)* 81: 6466 (1984); D. J. Fink, et al., *Human Gene Therapy* 3: 11 (1992).

Viral vectors capable of transferring genes into non-dividing cells usually require the generation of high-titer viral stock in order to achieve high efficiency gene transfer into different cell types. In addition, whenever a different regulatory sequence is to be tested for optimal level of gene expression into primary cells, a new viral stock must to be made and tittered for every modification. All these involve very time-consuming experimental manipulations.

Still another concern relates to the application of viral vectors in human gene therapy. A number of studies have been carried out in primates to test the safety of retroviral vectors for introducing cells transduced with retroviral vectors into animals. Some of these animals have developed various forms of lymphoma. R. E. Donahue, et al., *J. Exp. Med.* 176: 1125 (1992). Additional safety features have been introduced into some of the newer versions of retroviral vectors, yet are not available for all types of viral vectors.

SUMMARY OF THE INVENTION

It therefore is an object of the present invention to provide a method for high efficiency gene transfer to achieve expression, stable as well as transient, in a wide spectrum of cell types, including primary cells from various mammalian species.

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It is also an object of the present invention to provide cell lines which, even if derived from primary mammalian cells, are characterized by an extended life in culture.

It is another object of the present invention to provide a readily implemented screening system for identifying sequences that influence in the expression of cloned genes in various primary cell types from different species.

In accomplishing these and other objectives, there has been provided, in accordance with one aspect of the present invention, a transfection vector comprising a synthetic polypeptide linked electrostatically to a DNA structural sequence, forming a polypeptide-DNA complex, where the polypeptide is comprised of (A) a polymeric chain of basic amino acid residues, (B) an NLS peptide and (C) a hinge region of neutral amino acids that connects the polymeric chain and the NLS peptide. The polymeric chain preferably is comprised of between 10 and 50 residues, which can selected from lysine, arginine and ornithine, for example, while the hinge region is comprised of between 6 and 50 amino acid residues selected, for example, from glycine, alanine, leucine and isoleucine. The NLS peptide preferably is located at the amino terminus of said polypeptide and the polymeric basic amino acid chain at the carboxyl terminus. Among exemplary NLS peptides are the SV40 large T antigen NLS sequence, the polyoma large T antigen NLS sequence, the adenovirus E1a NLS sequence, and the adenovirus E1b NLS sequence.

In accordance with another aspect of the present invention an extended life cell line is provided that is the product of transfecting a mammalian cell with a vector as described above. The mammalian cell thus transfected can be selected, for example, from the group consisting of a human umbilical vein endothelial cell, a human dermal microvascular endothelial cell, a human peripheral blood monocyte/macrophage cell, a human aortic smooth muscle cell, and a rabbit liver non-parenchymal cell.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

PE

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention allows for the highly efficient transfer and stable integration of DNA into eukaryotic cells, such as cells from established mammalian cell lines, primary cells from mammalian tissues, and plant cells. The present invention also can be applied to developing cell lines from non-dividing cells, such as human peripheral blood monocytes and macrophages.

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In accordance with the present invention, a synthetic polypeptide is provided that can complex with a DNA molecule very efficiently by taking advantage of the high negative charge density on the polynucleotide. To this end, a synthetic polypeptide of the present invention comprises a DNA-binding sequence that is rich in basic amino acids, such as lysine, arginine and ornithine, and that is typically ten to fifty residues long. D-isomers of these basic amino acids are suitable so long as the length of the stretch of basic amino acids is within the prescribed length. The DNA-binding sequence can be a homopolymer of a basic amino acid, or it can comprise more than one kind of basic residue. The DNA binding sequence must be of adequate length to bind DNA, yet not so long that it precipitates out of the solutions employed in the present methodology, as discussed below.

A synthetic polypeptide of the present invention also contains an amino acid sequence corresponding to a nuclear localization signal (NLS) sequence. A representative sample from the diverse range of nuclear localization signals which have been identified are listed in Table I below. (SEQ ID NOS:1-54).

TABLE 1

Source	Nuclear Protein	Deduced Signal Sequence
Yeast	MATα2	(SEQ ID NO: 1) K—I—P—I—K
		(SEQ ID NO: 2) V—R—I—L—E—S—W—F—A—K—N—I
SV40	Large T	(SEQ ID NO: 3) P—K—K—K—R—K—V
Influenza virus	Nucleoprotein	(SEQ ID NO: 4) A—A—F—E—D—L—R—V—R—S
Yeast	Ribosomal protein L3	(SEQ ID NO: 5) P—R—K—R
Polyoma virus	Large T	(SEQ ID NO: 6) V—S—R—K—R—P—R—P—A
SV40	VP1	(SEQ ID NO: 7) A—P—T—K—R—K
Adenovirus	E1a	(SEQ ID NO: 8) K—R—P—R—P
SV40	VP2 (VP3)	(SEQ ID NO: 9) P—N—K—K—K—R—K
Prog	Nucleoplasmin	(SEQ ID NO: 10) R—P—A—A—T—K—K—A—G—Q—A—K—K—K—K—L—D—
Rat	Glucocorticoid receptor	(SEQ ID NO: 11) K—K—K—I—K
Monkey	v-sis (PDGF B)	(SEQ ID NO: 12) R—V—T—I—R—T—V—R—V—R—R—P—P—K—G—K—H—R—K
Yeast	Histone 2B	(SEQ ID NO: 13) G—K—K—R—S—K—A
Chicken	v-rel	(SEQ ID NO: 14) K—A—K—R—S—K—A
Influenza	NS1	(SEQ ID NO: 15) D—R—L—R—R
		(SEQ ID NO: 16) P—K—Q—K—R—K
Prog	N1	(SEQ ID NO: 17) V—R—K—K—R—K—T
		(SEQ ID NO: 18) A—K—K—S—K—Q—E
Human	c-myc	(SEQ ID NO: 19) P—A—A—K—R—V—K—L—D
		(SEQ ID NO: 20) R—Q—R—R—N—E—L—K-4-S—F
Human	lamin A	(SEQ ID NO: 21) T—K—K—R—K—L—E
HTLV-1	Rex($p27^{rex-III}$)	(SEQ ID NO: 22) P—K—T—R—R—R—P
		(SEQ ID NO: 23) S—Q—R—K—R—P—P
Adenovirus	pTP	(SEQ ID NO: 24) R—L—P—V—R—R—R—R—R—V—P
HIV-1	Tat	(SEQ ID NO: 25) G—R—K—K—R
Prog	Lamin L_1	(SEQ ID NO: 26) V—R—T—T—K—G—K—R—K—R—I—D—V

TABLE 1-continued

Rabbit	Progesterone receptor	(SEQ ID NO: 27) R—K—F—K—K
HIV-1	Rev	(SEQ ID NO: 28) R—R—N—R—R—R—R—W
Human	PDGF A-chain	(SEQ ID NO: 29) P—R-3-S—G—K—K—R—K—R—K—R—L—K—P—T
[Mouse	c-abl	(SEQ ID NO: 30) K-K-K-K-K]
Mouse	c-abl	(SEQ ID NO: 30) S-A-L-I-K-K-K-K-M-A-P
Adenovirus	DBP	(SEQ ID NO: 31) P—P—K—K—R
		(SEQ ID NO: 32) P—K—K—K—K—K
Chicken	c-erb-A	(SEQ ID NO: 33) S—K—R—V—A—K—R—K—L
Human	c-myb	(SEQ ID NO: 34) P—L—L—K—K—I—I—Q
Human	N-myc	(SEQ ID NO: 35) P—P—Q—K—K—I—K—S
Human	p53	(SEQ ID NO: 36) P—Q—P—K—K—K—P
Human	Hsp 70	(SEQ ID NO: 37) P—K—R—K—H—K—K—D—I—S—Q—N—K—R—A—V—R—R
Hepatitis B virus	Core protein	(SEQ ID NO: 38) S—K—C—L—G—W—L—W—G
Chicken	Ets1	(SEQ ID NO: 39) G—K—R—K—N—K—P—K
Yeast	Ribosomal protein L29	(SEQ ID NO: 40) K—T—R—K—H—R—G
		(SEQ ID NO: 41) K—H—R—K—H—P—G

Protein	Nuclear Localization Signals
TGA-1A (tobacco)	(SEQ ID NO: 42) [redacted]—L—A—Q—N—R—E—A—A—[redacted]—S—R—L—[redacted]
TGA-1B (tobacco)	(SEQ ID NO: 43) [redacted]—A—R—L—V—R—N—R—E—S—A—Q—L—S
	(SEQ ID NO: 44) [redacted]—Q—[redacted]
O2 NLS B (maize)	(SEQ ID NO: 45) [redacted]—E—S—N—R—E—S—A—[redacted]—S—[redacted]—[redacted]
NIa (Polyvirus)	(SEQ ID NO: 46) [redacted]—N—Q—K—H—K—L—K—M-32aa-[redacted]
VirD2 (Agrobacterium)	(SEQ ID NO: 47) [redacted]—P—R—E—D—D—D—G—E—P—S—E—[redacted]—E—[redacted]
VirE2 NSE1 (Agrobacterium)	(SEQ ID NO: 48) [redacted]—R—P—E—D—R—Y—I—Q—T—E—[redacted]—Y—G—[redacted]
VirE2 NSE2 (Agrobacterium)	(SEQ ID NO: 49) K—T—K—Y—G—S—D—T—E—I—K—L—L—S—K
O2 NLS A (maize)	(SEQ ID NO: 50) M—E—E—A—V—T—M—A—P—A—A—V—S—S—A—V—V—G—D—P
	(SEQ ID NO: 51) M-3-Y—N—A—I—L—[redacted]—L—E—E—D—L—E
R NLS A (maize)	(SEQ ID NO: 52) G—D—[redacted]—A—A—P—A—[redacted]—P
R NLS M (maize)	(SEQ ID NO: 53) M—S—E—[redacted]—E—K—L
RNLS C (maize)	(SEQ ID NO: 54) M—I—S—E—A—L—[redacted]—A-1-G—[redacted]

See Garcia-Bustos et al., *Biochem. Biophys. Acta* 1071: 83 (1991), Raikhel, N., *Plant Physiol.* 100: 1627 (1992), and Citovsky, V. et al., *Science* 256: 1802 (1992), the contents of each of which are hereby incorporated by reference.

In the present invention, an NLS peptide, which typically is six to fifteen amino acids in length, facilitates transport of the associated DNA into the nucleus. Because the synthetic polypeptide promotes the transport of the transfected gene into the nucleus of the host cell, this method provides both highly efficient stable and transient gene expression. Once inside the nucleus, the introduced DNA is immediately available to the transcription machinery, and can be expressed transiently. Simultaneously, the introduced DNA is also in the process of getting integrated into the host chromosome to give rise to stable expression. Thus, the method of the instant invention can achieve both transient and stable expression of introduced DNA.

Transient gene expression results when the method of gene transfer results in the introduction of the DNA sequences into the nucleus in an non-integrated form. Transient transfection is measured 24 to 72 hours after transfection by assays that measure gene expression of the transfected gene(s). In contrast, stable expression of the encoded

protein results when the transferred DNA sequences are stably integrated into the chromosomal DNA of the target cell. Stable transfectants remain capable of expressing the transfected DNA after two weeks or greater following the method of the invention. Commonly used assays monitor enzyme activities of chloramphenicol acetyltransferase (CAT), LAC-Z, β-galactosidase (β-gal), β-glucuronidase (GUS), luciferase, or human growth hormone, each of which may be contained in the present invention.

The NLS domain of the synthetic peptide is based on known endogenous peptide sequences that were identified by reference to two criteria: (1) sufficient to redirect a cytoplasmic protein to the nucleus and (2) necessary for directing a nuclear protein to the nucleus. Methods for assessing an NLS peptide's ability to direct protein to the nucleus are known in the art. See Garcia-Bustos, et al., supra, Sandler et al., *J. Cell Biol.* 109: 2665 (1989), and Citovsky et al., supra, the respective contents of which are hereby incorporated by reference. For example, an NLS peptide or a natural protein containing an NLS is fused to an otherwise non-nuclear protein, by either synthetic or recombinant production. The hybrid protein is then assessed for its ability to target the non-nuclear protein to the nucleus.

The presence of the non-nuclear protein in the nucleus can be determined by a functional assay or immunofluorescence. An illustrative assay entails the histochemical determination of a product produced by the non-nuclear protein, such as a colorimetric marker produced by β-gal or GUS. (A "colorimetric marker" includes an enzyme that can catalyze a reaction with a substrate to elicit a colored product which can be detected or measured by a variety of means, such as standard fluorescence microscopy, flow cytometry, spectrophotometry or colorimetry. "Immunofluorescence" relates to detecting the presence of the non-nuclear protein in the nucleus by means of an antibody specific for the targeted protein.)

In the past NLS peptides have been studied to assess their ability to target reporter proteins to the nucleus. Also, endogenous proteins containing an NLS, such as the VirD2 and VirE2 of Agrobacterium, have been shown to mediate the transfer of the Agrobacterium single-stranded DNA intermediate T-strand to the plant cell nucleus endogenously. See Citovsky, et al., supra. There has been no suggestion heretofore, however, to use an NLS peptide to target a polynucleotide to the nucleus of a eukaryotic cell.

A preferred NLS domain contains a short stretch of basic amino acids like the NLS of the SV40 virus large T antigen (PKKKRKV) (SEQ ID NO:3), which is an NLS that has been shown to be effective in mammalian cells (basic residues are highlighted). Another preferred NLS domain consists essentially of short hydrophobic regions that contain one or more basic amino acids (KIPIK) (SEQ ID NO:1), which is like the NLS of mating type α2. The NLSs that transport DNA into the plant cell nucleus often are bipartite, which means that they are usually comprised of a combination of two regions of basic amino acids separated by a spacer of more than four residues (see stippled segments in Table I), such as the Xenopus nucleoplasmin (KRPAATKKAGQAKKKK) (SEQ ID NO:55).

The NLS peptide of the present invention can be designed to accommodate different host cells, both mammalian and plant cell hosts.

The method described here can suitably be modified to introduce genes into plant protoplasts using plant NLSs, such as those described by Raikhel (1989), supra.

The present gene transfer system is also capable of transferring foreign DNA into gymnosperms and angiosperms. Procedures for assessing the introduction of foreign DNA in plants are known to the art, such as those disclosed by Miki, B. L., et al., in METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY, B. R. Glick et al., eds. (CRC Press, 1993), and Gruber, M. Y. et al., id.

A synthetic polypeptide of the present invention thus is comprised of a DNA binding domain and an NLS peptide domain which are separated by a third element, a hinge region of neutral amino acid, to minimize stearic interference between the two domains. For this purpose, the hinge region ranges in length from about six to twenty-five amino acids, and contains a stretch of neutral small amino acids without any bulky hydrophobic or ionic side chains.

The NLS sequence can be located at either the amino terminus or the carboy-terminus of the synthetic peptide. The arrangement of the two domains, basic amino acid sequence and NLS sequence can be interchanged without affecting the high gene transfer efficiency. As indicated previously, such a synthetic polypeptide binds electrostatically to the DNA that is to be introduced into the target cell. The weight ratio of polypeptide to DNA in the resulting complex generally is in the range of 1:1 to 1:10; for example, 1 μg polypeptide to 1 to 10 μg of polynucleotide.

In accordance with the present invention, entry of the DNA-polypeptide complex into cells can be promoted by treating target cells with a hypertonic solution, followed by hypotonic treatment of cells in the presence of gene-peptide complex. See, for example, Okada and Rechsteiner, *Cell* 29: 33 (1982). A suitable hypertonic solution can contain both polyethylene glycol (PEG) and sucrose, preferably in the concentration of 0.3M–0.6M and 10% to 25%, respectively, and is referred to as "primer" hereinafter. Okada et al., supra, and T. Takai, et al., *Biochem. Biophys. Acta* 1048: 105 (1990).

The methodology of the present invention has been used to develop stable transfectants of different established cell lines. It also has been employed to transfer genes into primary cells from different mammalian species, thereby to obtain cell lines that retain many of the characteristics of the cognate primary cells. Cell lines developed from primary cells via the methodology of the present invention are called "extended life" cell lines in this description, because the cell lines so developed retain almost all of the characteristics of their cognate primary cells even in their late passage. The range of cell types that can be converted to extended life cell lines, according to the present invention, is based on the availability of primary cells or the ability to isolate a primary cell from the organ in question. In this regard, the inventive methodology is not limited to cell types amenable to transformation. In addition to the cell types already mentioned, the present invention can be applied to pancreatic beta cells, human liver and kidney cells, and human hematopoietic stem cells, among others.

The methodology of the present invention has been used to develop an extended life cell line from human monocyte/macrophage cells, which are normally non-dividing. In all these instances, stable cell lines were obtained with a very high efficiency, either comparable to or better than the efficiency using retroviral vectors.

The present invention finds application as well in both ex vivo and in vivo gene therapies, where genetic material is transferred into specific cells of a patient. Ex vivo gene therapy entails the removal of the relevant target cells from the body, transduction of the cells in vitro, and subsequent reintroduction of the modified cells into the patient.

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A gene therapy pursuant to the present invention could involve an ex vivo introduction, into a particular cell type from the patient, of a polynucleotide coding for a correcting protein which can be produced in functional form by the targeted cell type. Genes suitable for expression in this regard include an adenosine deaminase gene, a globin gene, an LDL receptor gene, and a glucose cerebrosidase gene.

Different kinds of gene-therapy applications require either stable or transient gene expression. The method of the present invention is advantageous in that it can be used in gene therapy requiring either stable gene expression or transient gene expression. Transient expression of a foreign gene is preferred when expression of the exogenous product is needed only for a short period of time; thereafter, rapid clearance of the gene product and its vector is desirable. Transient expression is also desirable when the prolonged effects of the exogenous protein's expression are unknown. Stable expression in gene therapy is needed when the patient has a genetic defect that is incompatible with life. Such genetic defects include but are not limited to cystic fibrosis, Tay Sachs and cancer. Mulligan, *Science* 260: 926 (1993).

A gene therapy pursuant to the present invention also could involve an in vivo introduction of a structural DNA into cells of a patient's body. For stable transfer of genes into a target tissue using this method, the ligand to the target receptor will be conjugated to the synthetic polypeptide. The polypeptide-ligand combination can be complexed to a polynucleotide coding for the needed protein and then introduced into the host organism through blood circulation. When this complex reaches the target tissue, the whole complex will be taken up by cells containing the corresponding receptor for the ligand through receptor mediated process. Because of the NLS in the polypeptide-ligand complex, the complex will enter into the nucleus, resulting in a stable integration of the introduced gene into the host chromosome and, thereby, a correction of the genetic defect in the host. Cell-specific receptors are well known to those of skill in the art, as are their ligands which can be used in complexes for receptor-mediated gene transfer. Michael, S. I., et al., *J. Biol. Chem.* 268: 6866 (1993). For example, when the liver is the tissue targeted for gene therapy, the DNA encoding corrective protein is complexed to a synthetic neoglycoprotein that will target the complex to the asialoglycoprotein receptor on hepatocytes. For example, a cell type specific receptor such as asialoglycoprotein can be chemically linked to the transfection vector at the carboxyl terminal of the synthetic polypeptide molecule to deliver the foreign gene directly into liver cells. An additional hinge region can be incorporated into the molecule before chemically linking the polypeptide molecule to a cell-type specific ligand molecule, such as asialoglycoprotein or a cell-specific monoclonal antibody.

An example of a carrier useful for receptor-mediated gene transfer to liver is a synthetic glycoprotein in which bovine serum albumin (BSA) is covalently bound to poly L-lysine using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Ferkol et al., *FASEB* 7: 1081 (1993). To produce a neoglycoprotein conjugate for use in targeting DNA to liver, a reaction mixture that contains about 170 mM galactose, 4 mM poly (L-lysine), 160 mM BSA and 10 mM EDC (pH 7.5) can be incubated for 48 hours at 22° C. DNA is complexed to the neoglycoprotein carrier in a 360:1 molar ratio. The carrier-DNA complexes are dialyzed against 150 mM sodium chloride before transfection.

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Expression of a functional protein after transfection with DNA complexed to ligand alone is often transient. Ferkol et al., supra. The method of the present invention greatly improves the cell-specific targeting of receptor-mediated transfection by providing stable expression by increasing stable integration of a foreign DNA in the host cell using a synthetic polypeptide molecule of the present invention.

A variation of receptor-mediated gene transfer employs coupling a synthetic polypeptide as described above to monoclonal antibodies which recognize a cell surface antigen on the target cells. Maruyama et al., *Proc. Nat'l Acad. Sci. USA* 87: 5744 (1990). The coupled monoclonal antibody and synthetic polypeptide then are complexed with a DNA encoding the required or desired protein. This complex will target the DNA to the cells expressing the corresponding cell surface antigen. Any tissue of the human body can be targeted for the gene therapy of the present invention using the disclosed methods. A target tissue is suitable in this context so long as it is susceptible to genetic modification according to the present invention.

The present invention is further described with reference to the following examples, which are only illustrative and not limiting of the invention.

EXAMPLE 1

Transfer of Genes into Established Cell Lines for Purposes Transient Gene Expression and Selection of Stable Transfectants, Respectively

Forming the DNA-Polypeptide Complex and Transfecting Cells Therewith

The DNA or other polynucleotide to be transfected, such as a plasmid containing a gene for a drug resistance marker or coding a protein needed for expression in the host cell, is complexed to a synthetic polypeptide molecule in different weight ratios in an isotonic buffer solution. For example the weight ratio of DNA:polypeptide can be between 1:1 and 10:1, although ratios outside of this range may be evaluated empirically for achieving the objects of the present invention. An isotonic buffer solution such as Hanks buffered salt solution or HEPES buffered saline may be used for complexing DNA to polypeptide.

While the complex is formed, the cells that are to be transfected either remain attached to a substratum, such as a tissue culture dish, or are pelleted (for cells that grow in suspension). The cells are treated with a hypertonic primer solution, such as a concentration of 0.3M–0.6M sucrose and 10% PEG in either Tris-HCl or HEPES (pH 7.2) buffered solution, for 3–5 mins at room temperature. The primer solution then is removed.

After the DNA-polypeptide complex is formed, it is made hypotonic. The complex solution is hypotonic when it has a lesser osmotic pressure than a 0.15M or 0.9% solution of NaCl. For example, the complex in isotonic buffer can be made 40–55% hypotonic or 0.075M simply by adding an amount of distilled water that is equal to the volume of the complex in isotonic buffer. The hypotonic complex solution then is added to the cells that have been treated with the primer solution. Cells remain in the hypotonic DNA-polypeptide solution for 3–4 minutes. Fresh medium then is added to the cells to rinse away excess DNA-polypeptide solution. Thereafter, the cells are grown normally.

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Producing a Synthetic Polypeptide Molecule

An example of an synthetic polypeptide molecule of the present invention is one consisting of the amino acid sequence PKKKRKVSGGGGGKKKKKKKKKKKK(SEQ ID NO:56). Such a peptide can be synthesized, using standard methods of peptide production, and purified by standard methods using high pressure liquid chromatography (HPLC).

Selection of Stably Transfected Cell Lines

Transfected cells are grown in regular growth medium for 48 hours, and then plated in selective medium containing 400 μg/ml of G418. Cells were plated at a density of 100–1000 cells per 60 cm^2 dish. The number of G418-resistant colonies was determined two weeks after the initiation of selection. Other selectable markers, such as pHyg, may be used to achieve the results of the instant invention. K. Blochlinger, et al., *Mol. Cell. Biol.* 4: 2929 (1984).

This method gave a stable-transfection efficiency of 5–10%. Similar results were obtained using either G418 or hygromycin selection. In general the stable transfection efficiency achieved by the method of the instant invention is a few orders of magnitude greater than prior art methods. The instant invention's5–10% efficiency is several orders of magnitude better than the efficiency of the DNA-$CaPO_4$ co-precipitation method and at least equal or 5 times greater than the fairly high 1–10% level of stable transfection efficiency achieved by viral based methods.

TABLE II

Method	Stable Transfection Efficiency
Peptide-Mediated Gene Transfer	5–10%
Retroviral vectors	1–10%
Non-viral methods (e.g., $CaPO_4$)	<2%

That the transfectants of the instant invention are stable is shown by the following example. When G418 resistant colonies were grown without selection for variable period of times, and then tested for resistance to the antibiotic by plating the cells under clonal conditions, the same number of colonies were obtained both with and without G418. This result indicates that, once the cells are selected for the expression of the Neo gene, the resistance gene was retained stably in the chromosome.

Three different cell lines were used to test the efficiency of gene transfer of the new method. Mouse fibroblast cell line (L cells), mouse erythroleukemia cell line (C19TK), and COS cells. The COS cell line was used to establish conditions for transient gene expression. The eukaryotic expression vector, CH110, contains bacterial β-gal and was employed in these studies. The β-gal gene in CH110 is under the control of SV40 virus early promoter.

The COS cells were treated with primer and then exposed to DNA-polypeptide (2.5–5.0 μg) complex under hypotonic conditions. After this treatment, cells were returned to the normal growth condition. Transfected cells were grown at 37° C. for 48 hrs, and stained for the expression of the β-gal reporter gene. Forty to fifty percent of the cells were positive for the expression of the reporter gene.

Mouse L cells were transfected with eukaryotic expression vector containing the Neo gene, which codes for the antibiotic G418 resistance gene. L cells are sensitive to G418 at 400 μg/mL. Cells plated in 24-well tissue culture plates were then transfected with synthetic polypeptide complexed to the plasmid pRSV-Neo via the methodology of the present invention.

A mouse erythroleukemia cell line, C19TK, also was used as a representative cell line for testing the transfection efficiency of the present invention with respect to hematopoietic cells. The expression vector, pDR2, which carries a hygromycin-resistance gene, was used for these studies. C19TK cells are exquisitely sensitive for the antibiotic hygromycin. This cell line grows in suspension and, hence, was transfected in suspension.

Briefly, about million cells are spun down and the cell pellet is treated with primer. The cells are then exposed to DNA-polypeptide complex under hypotonic condition. Forty-eight hours after transfection, a known number of cells are plated in microtiter plates with hygromycin. The number of wells with growing population of cells was enumerated to determine the transfection efficiency. The stable transfection efficiency was about 1–5%, as compared to most of the other non-virus-based methods that are very poor. Thus, the method described herein is very efficient for stable transfection efficiency both for hematopoietic and non hematopoietic cell lines. Only some retrovirus based vectors give a transfection efficiency comparable to the efficiency obtained with the current method for hematopoietic cell lines. See Gilboa, et al. (1986), Miller, et al. (1986), Stuhlmann, et al. (1989), Miller, et al. (1989), and Zwiebel, et al. (1989), each cited above.

EXAMPLE 2

Transfer of Genes into Human Primary Cells

The gene transfer method of the present invention was used to generate extended life cell lines from different human primary cells. Most of the primary cells have a limited in vitro life span. The following cell types were employed to test the efficacy of the inventive method to generate extended-life cell lines by transfer of various oncogenes, either singly, in pairs of combinations, or combinations of more than two oncogenes. Rhim, J. S., et al., *Oncogene* 4: 1403 (1989).

EXAMPLE 3

Production of Extended Life Cell Lines

The method of introducing genes into primary cells is the same as that described above for introducing genes into established cell lines, such as the mouse fibroblast cell line L cells and the mouse erythroleukemia cell line C19TK. The main difference is that the host cell is a primary cell isolated from different species, human or other mammalian species, and the primary cells have only a limited in vitro life span. The isolation of primary cells from various tissue sources are well known to those of skill in the art.

In order to extend the life of primary cells that are endogenously incapable of extended growth in vitro, the cells are transfected with different oncogenes, such as SV40 large T antigen, polyoma large T antigen, adenovirus E1A and E1B, v-fms, Bc12, myc and ras. The oncogenes can be used either alone, in pairs of various combinations, or in combinations of more than two oncogenes.

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In addition, other genes that do not come under the category of oncogenes may be used. For example, genes that are important for DNA synthesis and normally active during the S phase of the cell cycle, such as the dihydrofolate reductase gene (DHFR), thymidine kinase gene, thymidylate synthetase gene, a DRTF1/E2F transcription factor encoding DNA, or DNA encoding the E2F transcription factor can be complexed to synthetic polypeptide and used to extend the life of primary cells. The human DHFR gene complexed to synthetic polypeptide can be introduced into primary cells to produce extended life cell lines. DNA encoding a transcription factor that is active during the S phase of the cell cycle are particularly useful in the method of the instant invention. La Thangue, N. B. *Trends in Biochemical Sciences* 19: 108 (1994); Johnson, D. G. et al., *Nature* 365: 349 (1993), the respective contents of which are hereby incorporated by reference.

Because untreated primary cells have only a limited life span in vitro, their ability to grow continuously in culture after treatment with the present invention served to select for extended life cell lines. No other drug selection markers need to be used to select for extended life cell lines derived from primary cells.

To produce extended life cells lines from primary cells, newly cultured primary cells were treated by the method of the present invention employing synthetic polypeptide conjugated with various oncogenes, such as SV40 large T antigen and/or Adeno E1A. The treated cells were plated in their appropriate growth media and passed after the cells reached confluency. A parallel set of a control untreated primary cells were cultured under the same growth conditions. Typically, control primary cells stop growing after about 4–10 passages, depending upon the cell type (cell split ratio was usually 1:4 by surface area). In contrast, continuously growing cell lines were obtained from different primary cell types described in the following examples.

EXAMPLE 4

Analysis of Transformed (Extended Life) Cells

Extended life cell lines containing the oncogene are identified by restriction cleavage, Southern analysis and/or Northern analysis using appropriate DNA probes.

The DNA of each transformed extended life cell line is analyzed by Southern hybridization to determine whether the cell lines carry the oncogenes used to establish such extended life cell lines. DNA is extracted from the cell lines and the nucleic acid pellet is re-suspended in 200 µl of 10 mM Tris-Cl pH 7.4, 0.1 mM EDTA, and 10 µg is digested with a specific restriction enzyme, electrophoresed through 1.0% agarose, and transferred to nitrocellulose. Southern, *J. Mol. Biol.* 98: 503 (1975). Filters are hybridized to a radioactively labelled DNA, encoding each of the oncogenes that gave rise to the corresponding extended life cell line, in the presence of 10% dextran sulfate. After overnight hybridization, the filters were washed twice in 2 X SSC, 0.1% SDS at 64° C.

Each transformed extended life cell line is analyzed by Northern hybridization to determine whether the cell lines transcribe the oncogenes. Cells not containing the oncogene of interest will not demonstrate transcripts in a Northern analysis whereas cells containing the DNA of interest will demonstrate a detectable transcript. Also, an ELISA method

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was used to detect the presence of oncogene products in some of the extended life cell lines, using publicly available antibodies that recognize the corresponding oncogene protein.

The presence of SV40 large T antigen and adenovirus E1A gene products in the HUVEC extended life cell line, as detected by ELISA, are shown in table 2. Briefly, the cell line grown in a 96 well tissue culture plate is fixed with glutaraldehyde and paraformaldehyde. The cells are then treated with antibodies to the corresponding oncogenes. Thereafter, the cells are washed and then treated with a secondary antibody linked with to β-galactosidase. The cells are washed and then treated with a substrate for β-galactosidase. The reaction develops a product which is then measured using a microplate reader.

To determine whether the extended life cell line has maintained the parental cell line phenotype may be determined by a number of ways. Extended life cells lines containing the oncogene are assessed by Northern analysis using a DNA probes encoding a cell-specific protein. The cell-specific DNA probe is labeled with ^{32}P-dCTP by nick translation pursuant, for example, to Rigby et al., *J. Mol. Biol.* 113: 237 (1977). Northern hybridization indicates that the extended life cell line is capable of transcribing the cell-specific protein.

Also, the maintenance of the parental phenotype in cells lines established according to the present invention can be determined by a number of biochemical methods, such as ELISA and enzyme assays, that determine the presence or function of a protein specific to the parental cell line. An antibody recognizing a protein produced only bythe parental cell line can be used in an ELISA or immunofluorescence assay. Cell-specific markers are well known to those of skill in the art. For example, albumin is a marker for hepatocytes, insulin is a marker for pancreatic beta islet cells, factor VIII is a marker for endothelial cells, actin and myosin are markers for smooth muscle cells, and non-specific esterass is a marker for brain microglial cells. In Table II, the parental phenotype of the extended life endothelial cells produced by the present method of the invention was verified by several ELISAs to determine the expression of cell-specific endothelial markers. The parental phenotype of the monocyte/macrophage extended life cell lines produced by the present method was verified using a lysozyme enzyme assay to measure macrophage specific markers.

EXAMPLE 5

Human Umbilical Vein Endothelial Cells

Endothelial cells isolated from the human umbilical vein can only be cultured for a limited of passages, usually five to six. These cells were transfected with a combination of oncogenes, SV40 large T antigen and adenovirus E1A, or with another combination of genes. At least two oncogenes are needed to develop a truly transformed cell line. Ruley, H. E., et al., *Nature* 304: 602 (1983). For the instant invention, SV40 large T antigen combined with v-myc or ras or some an other oncogene can be used. When the gens encoding SV40 large T antigen is combined with either adenovirus E1A or E1B genes in the method of the instant invention, extended life cell lines may be produced from human umbilical vein endothelial cells. E1A or E1B or SV40 large T antigen alone did not give rise to established cell line with the high frequency obtained from using SV40 large T

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antigen in combination with E1A or E1B. Synthetic polypeptide complexed to DNA encoding either the SV40 large T antigen or polyoma large T antigen combined with the E2F1 transcription factor gens also produces extended life HUVEC cells lines.

Since the non-transfected primary cells normally grow in vitro only for a limited population doublings, cells that have taken up the oncogenes capable of generating extended life span cell lines were selected simply by repeated passage of the cells. When the transfected population of cells grows continuously, as compared to a control population of parental cells, it is reasonable to conclude that the oncogenes used are capable of generating extended life cells from a given cell type.

In HUVEC, for example, SV40 large T antigen and adenovirus E1A or E1B were effective in giving rise to a cell line. This cell line has now been growing in culture for 40 passages. In contrast, normal HUVECs stop growing by passage 7 or 8. Such cell lines arose with a high efficiency. It also is possible to generate cell lines using as few as a couple of hundred cells, grown either in a 24- or 48-well plate. These cells also have the same morphological appearance as the primary HUVEC and also display many of the biochemical properties characteristic of normal HUVEC.

Some of the properties that are characteristic of endothelial cells that were measured in the HUVEC extended life cell line are also listed in Table 2. These properties were also measured by ELISA using specific antibodies listed in the Table 2.

TABLE III

ELISA assay for the expression of ELAM-1, VCAM-1, ICAM-1, SV40 large T antigen and adenovirus E1A by extended life HUVEC line

	$O.D_{450}$	
Antibody	-IL-1	+IL-1
Control	0.071	0,069
Anti ELAM-1	0.212	1.016
Anti VCAM-1	0.146	0.520
Anti ICAM-1	0.422	1.524
Anti SV40 large T	0.618	—
Anti EIA	0.725	—

T150X

EXAMPLE 6

Human Cord Blood-Derived Monocyte Cell Line

Adherent cells from human cord blood cells were transfected with different combinations of oncogenes in suspension using the method of the present invention. The resulting cells are selected in Granulocyte-Macrophage Colony Stimulating Factor (G-CSF). Control cells did not grow in culture, whereas growing populations of monocytes were obtained with several combinations of oncogenes. One preferred combination of polyoma large T antigen and adenovirus E1B encoding DNA produced extended life monocyte cells lines with somewhat higher efficiency than other combinations. Another preferred combination of SV40 large T or polyoma large T antigen and the E2F1 transcription factor gene produces monocyte extended life cells with high efficiency. The monocyte extended life cells also display many of the properties of normal monocytes, which illustrates the utility of the present invention in generating cell lines of hematopoietic origin.

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EXAMPLE 7

Extended Life Human Aortic Smooth Muscle Cells

The method of the instant invention has also been used to generate extended life cell lines using a specific combination of oncogenes. Human aortic smooth muscle cells were obtained from Clonetics Corporation (San Diego, Calif. U.S.A.) and transfected with several combination of oncogenes. The combination of polyoma large T antigen and EiB gave rise to a continuously growing population of smooth muscle cells. Another preferred combination of SV40 large T or polyoma large T antigen and the E2F1 transcription factor gene produces extended life human aortic smooth muscle cells with high efficiency. This cell line resembles the early passage primary aortic smooth muscle cells morphologically. The extended life human aortic smooth muscle cells also express smooth cell actin and myosin well beyond passage 20.

EXAMPLE 8

Other Extended Life Cell Types

Primary cells from other species, such as rabbit and monkey, also have been used to generate cell lines. Transfection methods employed for primary cells from non-human species are similar to those used for human primary cells. When developing an extended life cell line from a new primary cell, several different combinations of available oncogenes should be tried. For example, at least five or six pairs of combinations of SV40 large T antigen, adenovirus E1A, adenovirus E1B, polyoma virus large T antigen or others available to those in the art. That combination of genes that gives rise to an extended life cell lines from a given primary cell type is determined as described in the above examples.

When the E2F1 transcription factor gens is complexed to synthetic polypeptide in combination with DNA encoding either the SV40 large T antigen or polyoma large T antigen, extended life cells lines can be produced from a variety of primary cell types, such as HUVEC, dermal microvascular endothelial cells, human aortic smooth muscle cells, and bone marrow monocyte/macrophage cells. Thus, the method of the present invention can identify a combination of oncogene DNAs that is highly efficient in producing extended life cells lines from the primary cells of various species. The present invention also comprehends a combination of an oncogene and an S-phase transcription factor gens which likewise is highly efficient in producing extended life cells lines from different types of primary cells.

EXAMPLE 9

Identification of Cell Type-Specific Transcriptional and Translational Regulatory Sequences

The present invention provides a screening system for identifying sequences that influence the expression of cloned genes in various primary cell types from different species. The instant invention can identify cell type specific transcription and translational regulatory sequences. The sequence in question typically will be cloned into a vector containing a reporter gens, such as chloramphenicol acetyl transferass or luciferass, and then transfected into various cell types using the method described herein. Expression of the reporter gens determines the tissue specificity of the regulatory sequence.

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